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The effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on milk production, nitrogen utilization, and amino acid metabolism of lactating dairy cows

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ABSTRACT

Alternative protein sources such as microalgae and faba beans may have environmental benefits over rapeseed. We studied the effects of rapeseed meal (RSM) or faba beans (FB) as a sole protein feed or as protein feeds partially substituted with *Spirulina platensis* (spirulina) microalgae on milk production, N utilization, and AA metabolism of dairy cows. Eight multiparous Finnish Ayrshire cows (113 ± 36.3 d in milk; mean ± SD) were used in a balanced, replicated 4 × 4 Latin square with 2 × 2 factorial arrangement of treatments and 21-d periods. Four cows in one Latin square were rumen cannulated. Treatments were 2 isonitrogenously fed protein sources, RSM or rolled FB, or one of these sources with half of its crude protein substituted by spirulina (RSM-SPI and FB-SPI). Cows had ad libitum access to total mixed rations consisting of grass silage, barley, sugar beet pulp, minerals, and experimental protein feed. The substitution of RSM with FB did not affect dry matter intake (DMI) but decreased neutral detergent fiber intake and increased the digestibility of other nutrients. Spirulina in the diet decreased DMI and His intake. Spirulina had no effect on Met intake in cows on RSM diets but increased it in those on FB diets. Energy-corrected milk (ECM) and protein yields were decreased when RSM was substituted by FB. Milk and lactose yields were decreased in cows on the RSM-SPI diet compared with the RSM diet but increased in those on FB-SPI compared with FB. The opposite was true for milk fat and protein concentrations; thus, spirulina in the diet did not affect ECM. Feed conversion efficiency (ECM:DMI) increased in cows on FB diets with spirulina, whereas little effect was observed for those on RSM diets. The substitution of RSM by FB decreased arterial concentration of Met and essential AA. Spirulina in the diet increased milk urea N and ruminal NH_4-N and decreased the efficiency of N utilization in cows on RSM diets, whereas those on FB diets showed opposite results. Met likely limited milk production in cows on the FB diet as evidenced by the decrease in arterial Met concentration and milk protein yield when RSM was substituted by FB. The results suggest the potential to improve milk production response to faba beans with supplementation of Met-rich feeds such as spirulina. This study also confirmed spirulina had poorer palatability than RSM and FB despite total mixed ration feeding and lower milk production when spirulina partially replaced RSM.
Key words: microalgae, *Spirulina platensis*, rapeseed meal, faba bean, dairy cow

INTRODUCTION

Sustainable feed production and feeding are key factors in achieving resource-efficient and resilient livestock production systems with minimal environmental load and high product quality (Makkar, 2016). A sustainable animal diet is characterized as a diet that is balanced in all nutrients and free from harmful components, meets production objectives, generates animal products safe for human consumption, and integrates the planet, people, profit, and ethical dimensions of sustainability (Makkar and Ankers, 2014). Sustainability concerns are related to current rapeseed cultivation practices because neonicotinoid insecticides commonly used in seed dressing of, for example, nonorganic rapeseed pose significant risks to many living organisms (Wood and Goulson, 2017). The recent restriction of neonicotinoids by the European Union but also the disruption of pollination services by neonicotinoids may in the near future impede the availability of rapeseed feed (Budge et al., 2015; Hokkanen et al., 2017), a high-quality protein source well suited to grass silage-based dairy cow nutrition (e.g., Huhtanen et al., 2011; Martineau et al., 2013).

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The production of legumes such as faba beans (**FB**; *Vicia faba*) has multiple environmental benefits, including low reliance on fossil-fuel-intensive mineral fertilizers; reduced N_2O emissions from soil; increased diversification in crop rotation, leading to richer biodiversity and lower pest and disease pressure; and improved soil fertility and carbon storage (Watson et al., 2017). Moreover, FB outyield other conventional protein feeds in northern European conditions (Laine et al., 2017). However, the protein value of FB may be impaired by their considerably high rumen degradability and low concentration of methionine (Aguilera et al., 1992; Duc et al., 1999; Luke, 2019), which is often the limiting AA for milk production (e.g., Pisulewski et al., 1996). Indeed, compared with rapeseed, FB have resulted in lower milk and protein yields in dairy cows (Puhakka et al., 2016; Ramin et al., 2017; Halmemies-Beauchet-Filleau et al., 2018). Previously, balancing the AA supply in metabolizable protein has increased milk and protein yields and improved the conversion of feed N into milk protein (N use efficiency, **NUE**; Haque et al., 2012), consequently leading to decreased N emissions to the environment. Thus, balancing the AA composition of FB with methionine supplementation or methionine-rich feed components may be beneficial in dairy cow diets.

Many microalgae species, including spirulina, are high in CP and methionine (Becker, 2013; Lamminen et al., 2017, 2019). Algae and different algae products are already included in the European Community Catalogue of Feed Materials [Commission Regulation (EU) No. 68/2013], so their usage is allowed for animal feeding purposes. The milk protein yield responses to spirulina in dairy cow nutrition have been slightly lower than those to rapeseed meal (**RSM**; Lamminen et al., 2017), but similar to soybean meal (Lamminen et al., 2019). However, feed intake problems are related to the feed use of microalgae when concentrates and forage are given separately (Lamminen et al., 2017, 2019). Providing feed components as TMR may improve the intake of microalgae diets by masking the flavor of unpalatable feed components. Moreover, this practice is known to decrease sorting behavior of animals, leading to more balanced nutrient intake (DeVries and von Keyserlingk, 2009; Greter et al., 2010).

The aim of this study was to investigate the effects of RSM or FB, as sole protein feed or a protein feed partially substituted by spirulina microalgae, on milk production and N utilization of dairy cows. We hypothesized that (1) the substitution of RSM by FB decreases milk yield and NUE; (2) the partial substitution of RSM or FB by spirulina in TMR feeding does not affect DMI; and (3) the milk production response to spirulina inclusion differs between the 2 protein sources in the basal diet with decreased milk yield in cows on RSM-supplemented diets and increased milk yield in cows on FB-supplemented diets.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

The experiment was conducted at the University of Helsinki research farm in Helsinki, Finland. All experimental procedures were approved by the National Animal Experiment Board in Finland in accordance with the guidelines established by the European Union Directive 2010/63/EU and the current Finnish legislation on animal experimentation (Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013). Eight multiparous Finnish Ayrshire cows averaging 113 ± 36.3 (mean ± SD) DIM and producing 33.9 ± 4.79 kg milk/d were used, of which 4 were rumen cannulated (100 mm i.d.; Bar Diamond Inc., Parma, ID). Cows were weighed on 2 consecutive days at the beginning (707 ± 63.2 kg) and at the end (713 ± 38.9 kg) of the experiment.

Cows were randomly allocated to 4 experimental treatments according to a replicated, balanced 4 × 4 Latin square design with four 21-d experimental periods and 2 × 2 factorial arrangement of treatments. Four experimental treatments consisted of RSM (Raimix Rypsi, Raisioagro Ltd., Raisio, Finland) or rolled FB as sole protein feed (100%) or RSM or FB with 50% of the CP substituted by spirulina (Duplaco B.V., Hengelo, the Netherlands; **RSM-SPI** and **FB-SPI**). According to the supplier, spirulina was produced in open raceway ponds, and after harvesting, it was centrifuged to increase DM content before drying in a dry tower. The isoenergetic experimental diets were isonitrogenous with regard to N supply from protein feed. The collective term microalgae is used in its broadest sense to describe both eukaryotic and prokaryotic microphytes. Cows had ad libitum access to water and TMR consisting of grass silage, rolled barley, experimental protein feed, sugar beet pulp (Raimix Leike, Raisioagro Ltd.), and mineral-vitamin mixture (Seleeni-E-Melli TMR, Raisioagro Ltd.). The concentrate-to-forage ratio of TMR was 43:57 in DM for all treatments, and this ratio was maintained between experimental treatments by adjusting the amount of barley in TMR. The experimental grass silage was preserved from a mixture of second cut timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*), prewilted and ensiled in bales with formic acid–based additive (76% formic acid, 5.5% ammonium formate; AIV 2 Plus, Taminco Finland Ltd., Oulu, Finland) applied at the rate of 7 L/1,000 kg. To improve aerobic stability of silage during feeding out,

1 L of 99.5% propionic acid (Propion, Hankkija Ltd., Hyvinkää, Finland) diluted with water at a rate of 1:8 (vol:vol) was added per 1,000 kg of TMR during mixing. To ensure even mixing of powdery algae on TMR, spirulina was first mixed with barley and water (0.34 L/kg of barley). A similar amount of water was also added to TMR mixtures containing no spirulina. The detailed ingredient and chemical composition of the TMR is given in Table 1, and the chemical composition of individual feed ingredients in Table 2. Cows were housed in individual tie stalls equipped with Roughage Intake Control system (Insentec BV, Marknesse, the Netherlands). Cows were milked twice daily at 0600 and 1700 h, orts were removed daily at 0800 h, and TMR was delivered to animals 3 times (at 0830, 1400, and 2000 h) daily.

Measurements, Sampling, and Chemical Analysis

Feed intake and milk yield of the cows were recorded throughout the experiment; however, only the measurements on d 15 to 21 of each period were used for statistical analysis. During this measurement period, representative samples of feeds were collected daily and combined by period to provide a composite sample for analysis of DM, ash, CP, NDF, indigestible NDF, crude fat, starch, AA, and acid-insoluble ash. Silage samples were also analyzed for pH, NH_3-N, VFA, lactic acid, ethanol, water-soluble carbohydrates, and in vitro digestible OM in DM (**DOMD**). The indigestible NDF concentration of spirulina was not analyzed because spirulina did not contain any NDF in the current experiment. Feed samples were analyzed using similar standard procedures as described previously (Lamminen et al., 2017). Silage DOMD was measured based on in vitro pepsin-cellulase solubility (Friedel, 1990) with the modifications of Nousiainen et al. (2003) and using cellulase enzyme Onozuka R-10 (Yakult Honsha Co. Ltd., Tokyo, Japan). The results of pepsin-cellulase solubility were converted to in vivo OM digestibility using correction equations of Huhtanen et al. (2006). For NDF analysis, crucibles with pore sizes of 40 to 100 μm were used for all samples. Results of NDF are expressed exclusive of residual ash, the DM content of silages was corrected for the loss of volatile compounds (lactic acid, VFA, NH_3-N, and ethanol) according to Huida et al. (1986), and CP content of feeds was calculated as Kjeldahl-N × 6.25. The use of the same N-to-CP conversion factor (6.25) for all experimental feeds was considered justified because the comparison of the N concentration calculated based on AA composition and the Kjeldahl-N concentration indicated no difference in nonprotein N between spirulina and other concentrate components. The total apparent digestibility of the diets and nutrients was determined using acid-insoluble ash as an internal marker (Van Keulen and Young, 1977). Nomenclature of International Union of Pure and Applied Chemistry (**IUPAC**) was used for the naming of AA. The terms Nπ (nitrogen atom closest to the side chain) and Nτ (nitrogen atom farthest from the side

Table 1. Ingredient and chemical composition (% of DM unless otherwise noted) of experimental TMR fed to dairy cows

	Treatment[1]			
Item	RSM	RSM-SPI	FB	FB-SPI
Ingredient composition				
Grass silage	56.9	56.9	56.9	56.9
Barley	26.0	28.1	23.8	27.0
Sugar beet pulp	6.49	6.49	6.49	6.49
Rapeseed meal	9.50	4.75		
Faba beans			11.7	5.88
Spirulina platensis		2.64		2.64
Mineral-vitamin supplement	1.12	1.12	1.12	1.12
Chemical composition				
OM	91.0	91.2	91.4	91.3
NDF	39.5	38.1	37.3	37.0
CP	16.5	16.9	16.6	17.0
Starch	16.7	17.9	19.1	19.1
Crude fat	3.44	3.44	3.16	3.31
ME, MJ/kg of DM	11.6	11.6	11.7	11.6
N from concentrates,[2] % of N	45.6	46.9	45.9	47.0
N from supplementary protein feed,[3] % of N	19.9	20.3	21.9	21.3

[1]RSM = rapeseed meal; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba bean; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed.

[2]Includes barley, sugar beet pulp, supplementary protein feed, and mineral-vitamin supplement.

[3]Includes rapeseed meal or faba beans or mixture of one these and *Spirulina platensis*.

chain) were used to describe the position of methylated nitrogen atoms in the imidazole ring of histidine, according to the IUPAC recommendations. Thus, 3-methylhistidine, the product of muscle actin and myosin catabolism, is referred to as Nτ-methylhistidine, and 1-methylhistidine, the product of anserine breakdown, is referred to as Nπ-methylhistidine.

A detailed description of sampling and analysis of rumen fluid, blood, milk, feces, and urine is given elsewhere (Lamminen et al., 2017). Briefly, rumen liquid samples of 100 to 150 mL from rumen cannulated cows were collected on d 20 of each period at 0600, 0730, 0900, 1030, 1200, 1330, 1500, and 1630 h via the rumen cannula and analyzed for pH, VFA, and NH_4-N. Blood samples were collected from the superficial epigastric (mammary) vein and coccygeal (tail) vessel of all cows on d 21 of each period at 0530, 0830, and 1130 h and analyzed for acetic acid, as in Halmemies-Beauchet-Filleau et al. (2017), and BHB, glucose, insulin, nonesterified fatty acids, AA, and carnosine, as in Lamminen et al. (2017). Milk samples were collected from all experimental cows over 4 consecutive milkings, starting on d 18 at 1700 h, and analyzed for fat, protein, lactose, and MUN. Spot samples of feces were obtained from the rectum of each cow on d 17 to 20 of each period at 0700 and 1530 h, composited by cow within period, and analyzed for DM, ash, N, NDF, acid-insoluble ash, and starch. Spot samples of urine (minimum of 500 mL) were obtained by mild manual stimulation of the vulva on d 18 at 0530 and 1430 h and on d 19 at 1000 and 1900 h and analyzed for N, urea-N, and purine derivatives (allantoin, creatinine, and uric acid). The BCS of cows was assessed using a scale of 1 to 5 according to Edmonson et al. (1989) by the same 2 observers at

Table 2. Chemical composition (% of DM unless otherwise noted) of the experimental feeds

Item	Silage[1]	Barley	Sugar beet pulp	M-V supplement[2]	Rapeseed meal	Faba beans	*Spirulina platensis*
DM, %	29.3	87.3	87.8	99.4	87.7	87.2	94.6
Ash	10.7	2.5	7.1	88.5	8.4	4.3	6.8
CP	15.9	12.1	16.9		34.5	31.0	68.3
NDF	50.5	20.1	35.8		31.8	15.8	0
Indigestible NDF	5.82	4.08	12.9		16.7	0.322	
Starch	NA[3]	59.9	13.0		3.20	34.6	3.49
Crude fat	3.78	2.61	3.44		4.11	1.50	5.63
ME, MJ/kg	10.9	13.2	12.8		11.4	12.8	10.9
EAA, % of CP							
Arg	2.65	5.47	5.69		5.58	9.25	7.14
His	1.14	2.08	2.35		2.13	2.28	1.47
Ile	3.17	3.49	3.50		3.76	3.77	5.29
Leu	5.61	6.50	6.12		6.49	6.62	8.23
Lys	2.84	3.68	4.33		4.80	5.59	4.49
Met	1.08	1.20	1.45		1.56	0.522	1.83
Phe	3.65	4.88	3.65		3.59	3.72	4.18
Thr	2.81	3.27	3.98		4.32	3.15	4.66
Trp	0.828	1.09	1.17		1.14	0.710	1.15
Val	4.09	4.79	4.62		4.77	4.14	5.90
NEAA, % of CP							
Ala	5.15	4.00	4.10		4.16	3.64	7.25
Asp	5.45	6.02	6.54		6.78	9.64	9.04
Cys	0.873	2.43	2.54		2.58	1.36	0.926
Glu	6.37	22.6	15.5		15.6	15.2	13.3
Gly	3.36	3.83	4.37		4.54	3.69	4.52
Pro	3.89	10.06	5.16		5.45	3.75	3.45
Ser	2.43	4.10	4.13		4.11	4.35	4.54
Tyr	2.41	2.93	2.93		2.85	2.91	4.10
Σ Branched AA[4]	12.9	14.8	14.2		15.0	14.5	19.4
Σ EAA	27.9	36.4	36.9		38.2	39.8	44.4
Σ NEAA[5]	29.9	55.9	45.2		46.0	44.5	47.1
Σ Total AA[6]	57.8	92.4	82.1		84.2	84.3	91.5

[1]In DM: 0.395% of ethanol, 3.59% of lactic acid, 0.952% of acetic acid, 0.592% of propionic acid, butyric acid not detected, 9.08% of water-soluble carbohydrates, 9.52% N of NH_3, 5.82% of indigestible NDF, 68.1% of in vitro digestible OM, pH 4.84.

[2]Mineral-vitamin (M-V) supplement; contained 199 g/kg of Ca, 115 g/kg of Na, 0.68 g/kg of Mg, 0.01 g/kg of P, 1,635 mg/kg of Zn, 737 mg/kg of Cu, 558 mg/kg of Mn, 65.5 mg/kg of I, 35 mg/kg of Se, 34 mg/kg of Co, 1,220 mg/kg of vitamin E, 372,000 IU/kg of vitamin A, and 93,000 IU/kg of vitamin D_3.

[3]Not analyzed.

[4]Includes Ile, Leu, and Val.

[5]Includes NEAA listed in the table.

[6]Σ EAA + Σ NEAA.

the beginning of the experiment and the end of each experimental period, and calculated as averages of the 2 observers.

Calculations and Statistical Analysis

Individual daily DMI was calculated as the difference between DM offered and DM residue. Energy-corrected milk yield was calculated according to Sjaunja et al. (1991). The ME content of concentrate components was estimated based on the following equation (Ministry of Agriculture, Fisheries and Food, 1984):

$$\text{ME (MJ/kg DM)} = [15.2 \times \text{digestible CP (g/kg of DM)} + 34.2 \times \text{digestible crude fat (g/kg of DM)} + 12.8 \times \text{digestible crude fiber (g/kg of DM)} + 15.9 \times \text{digestible N-free extract (NFE; g/kg of DM)}]/1{,}000.$$

The crude fiber concentration of spirulina was assumed to be zero based on zero NDF concentration in the current experiment, and the NFE concentration of all feeds was calculated by difference of other nutrients. The digestibility coefficients of spirulina were based on Hintz et al. (1966) reporting ruminant digestibility values for mixture of several microalgae grown on sewage, and those of barley and FB were based on Finnish feed tables (Luke, 2019). The ME concentration of RSM was based on the information given by the feed manufacturer and was estimated using the same equation as other concentrate components. The ME concentration of silage was calculated by multiplying in vitro DOMD concentration by 0.016 (Luke, 2019). Requirements of ME (MJ/d) for maintenance and milk production were calculated as

$$\text{BW (kg)}^{0.75} \times 0.515 + \text{ECM yield (kg/d)} \times 5.15 \text{ (Luke, 2019).}$$

The intake of ME was corrected for feeding level and associative effects of feeds (Luke, 2019). The ME balance of animals was calculated as the difference of corrected ME intake and ME requirements. For the calculation of human-edible efficiency of milk production (ratio of milk N to human-edible N intake), the interpretation of Wilkinson (2011) about the human-edible proportions of different feeds and by-products was used. Thus, the human-edible proportions were assumed to be 0% for grass silage and vitamin-mineral mixture, 20% for sugar beet pulp and RSM, and 80% for barley and FB. Microalgae was not included in Wilkinson (2011), however, the human-edible proportion of intact and food-grade microalgae was assumed to be 80% in line with cereals and pulses.

The ruminal microbial CP production and daily urine volume were estimated from the excretion of purine derivatives in urine assuming the creatinine excretion rate of 25 mg/kg BW (Puhakka et al., 2016). The Fick principle, based on stoichiometric transfer of phenylalanine and tyrosine uptake into milk (Cant et al., 1993), was used to determine mammary plasma flow similarly to Vanhatalo et al. (1999). Mammary uptake of plasma metabolites and AA was then calculated as arteriovenous difference × mammary plasma flow.

Experimental data were subjected to ANOVA using the Mixed procedure of SAS 9.4 version (SAS Institute Inc., Cary, NC). The statistical model was as follows:

$$Y_{ijkl} = \mu + A(S)_i + S_j + P(S)_k + D_l + E_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, A is the effect of animal, S is the effect of square, P is the effect of period, D is the effect of experimental diet, and E is the residual error. Square, period within square, and diet were considered as fixed effects, and animal within square as a random effect. The measurements of rumen fermentation characteristics were subjected to ANOVA for repeated measures with the model as follows:

$$Y_{ijkl} = \mu + A_i + P_j + D_k + T_l + APD_{ijk} + AT_{il} + PT_{jl} + DT_{kl} + E_{ijkl},$$

where Y_{ijkl} is the dependent variable; μ is the overall mean; A is the effect of animal (random effect); P is the effect of period (fixed effect); D is the effect of experimental diet (fixed effect); T is the effect of sampling time (fixed effect); APD is the interaction of A, P, and D (random effect); AT is the interaction of A and T (random effect); PT is the interaction of P and T (fixed effect); DT is the interaction of D and T (fixed effect); and E is residual error. The degrees of freedom were calculated according to the Satterthwaite method. The covariate structure AR(1) was applied with the interaction of animal and period as the subject for repeated measures. In the presence of D × T interactions, data from individual sampling times were further statistically analyzed with a simplified model, with animal as random effect and period and diet as fixed effects. Otherwise, only least squares means of treatment effects on rumen fermentation characteristics are presented.

P-values ≤ 0.05 were regarded as significant, and $0.05 < P \leq 0.10$ was accepted as a tendency. Sums of squares of the treatment effects were further sepa-

rated into single degree of freedom comparisons using orthogonal contrasts. The contrasts were as follows: BP = the effect of protein source in the basal diet (RSM + RSM-SPI vs. FB + FB-SPI), SPI = the effect of partial substitution of RSM and FB by spirulina (RSM + FB vs. RSM-SPI + FB-SPI), and BP × SPI = interaction between the protein source in the basal diet and its substitution by spirulina.

RESULTS

Composition of Feeds

The chemical composition of experimental feeds is presented in Table 2. Grass silage had moderate CP and in vitro DOMD content, the concentrations of fermentation acids were low, and the concentration of residual water-soluble carbohydrates was high (see the footnotes of Table 2 for details). The chemical composition of FB differed from RSM and spirulina with markedly higher starch concentration. The CP concentration in spirulina was twice as high as in RSM and FB, and spirulina contained no detectable NDF. The concentration of methionine was the lowest in FB protein and the highest in spirulina. Spirulina protein had lower histidine and higher branched-chain AA (**BCAA**) concentrations in comparison to RSM and FB.

Feed and Nutrient Intake, Digestibility, and Milk Production

Compared with RSM, FB resulted in lower intakes of NDF and crude fat ($P < 0.001$) (Table 3). When half of the protein in RSM and FB was substituted by spirulina, the intake of DM, OM, NDF, and ME was decreased ($P < 0.05$). Cows' intake of starch was increased when they were on the RSM-supplemented diet when including spirulina, but decreased when they were on the FB-supplemented diet ($P < 0.01$ for interaction).

Compared with RSM, FB resulted in lower intakes of threonine, tryptophan ($P < 0.001$), and valine ($P < 0.01$), and it tended ($P < 0.10$) to result in lower intake of NEAA (Table 3). The opposite was true for lysine ($P < 0.05$). Spirulina inclusion in the diet increased the intake of isoleucine ($P < 0.01$), leucine, threonine, valine, and BCAA ($P < 0.05$), but it decreased the intake of histidine ($P < 0.05$). The intake of arginine was increased when cows were on the RSM-supplemented diet when including spirulina, but decreased when they were on the FB-supplemented diet ($P < 0.01$ for interaction). Spirulina inclusion did not affect methionine intake with the RSM-supplemented diet, the average intake being 47.9 g/d, but increased it with the FB-supplemented diet, from 38.5 to 43.4 g/d ($P < 0.01$ for interaction).

The apparent total-tract digestibility of DM, OM, and CP was higher ($P < 0.05$) for FB than RSM. The digestibility of starch was increased with RSM, but decreased with FB-supplemented diets when including spirulina ($P < 0.01$ for interaction).

Energy-corrected milk ($P < 0.01$) and protein ($P < 0.001$) yields were lower, and fat yield tended to be lower ($P = 0.074$) with FB-supplemented than with RSM-supplemented diets. Spirulina inclusion decreased milk and lactose yields from cows on the RSM-supplemented diet but increased those from cows on the FB-supplemented diet ($P < 0.01$ for interaction). Milk fat, protein, and MUN concentrations increased with RSM but decreased with the FB-supplemented diet when including spirulina ($P < 0.05$ for interaction). Spirulina inclusion in the diet increased the efficiency ratio of ECM:DMI associated with the FB-supplemented diet, whereas little effect was observed with RSM ($P < 0.05$ for interaction).

Energy and Nitrogen Metabolism

A significant interaction ($P < 0.05$) between sampling time and diet in rumen fermentation characteristics was observed only for pH. Therefore, only least squares means of the treatment effects are presented in Table 4, and the dietary effects on ruminal pH at each sampling time are illustrated in Figure 1. Faba bean diets resulted in lower rumen molar proportion of acetate than RSM diets ($P < 0.05$). Spirulina inclusion in the diet decreased rumen pH ($P < 0.05$) especially during the first hours of sampling (<6 h) ($P = 0.047$ for time × diet interaction; Figure 1). Spirulina also tended to increase total VFA concentration in the rumen ($P < 0.10$). The molar proportion of acetate ($P < 0.01$) decreased and that of propionate ($P < 0.05$) increased when spirulina substituted half of the protein source of the basal diet. The molar ratios of acetate to propionate and (acetate+butyrate) to propionate were also decreased when spirulina was included in the diet ($P < 0.05$). Spirulina inclusion in the diet increased ruminal NH_4-N concentration and molar proportion of isobutyrate in cows on RSM, but decreased those in cows on the FB-supplemented diet ($P < 0.05$ for interaction). The molar proportion of isovalerate was also increased in cows on RSM, whereas no effect was seen in those on FB ($P < 0.01$ for interaction).

Compared with RSM diets, FB diets resulted in lower N excretion in milk ($P < 0.001$) and feces ($P < 0.05$), and a lower proportion of N secreted in feces ($P < 0.05$). In contrast, the excretion of N in urine ($P < 0.05$) and the proportion of N secreted in urine ($P <$

0.01) were higher for FB than for RSM diets. Uric acid excretion in urine was increased ($P < 0.05$) when half of the protein in RSM and FB was substituted by spirulina. Spirulina inclusion in the diet also increased the excretion of N and urinary urea N in urine ($P < 0.05$), the proportion of N excreted in urine ($P < 0.01$), and total N excretion via urine and feces ($P < 0.05$), and it decreased N balances ($P = 0.01$). Spirulina inclusion in the diet decreased the proportion of N secreted in milk from cows on RSM, but increased that from cows on the FB-supplemented diet ($P < 0.05$ for interaction). Human-edible protein efficiency was decreased

Table 3. Effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on nutrient and ME intake, nutrient digestibility, milk yield, and milk composition in lactating cows

	Treatment[1]					Orthogonal contrast[2]		
Item	RSM	RSM-SPI	FB	FB-SPI	SEM	BP	SPI	BP × SPI
Intake, kg/d								
DM	23.3	22.8	23.1	22.3	0.57	0.20	0.037	0.50
OM	21.2	20.8	21.1	20.3	0.52	0.29	0.042	0.45
NDF	9.17	8.69	8.60	8.27	0.216	<0.001	0.002	0.47
Starch	3.91	4.09	4.42	4.24	0.106	<0.001	1.00	0.006
CP	3.86	3.88	3.84	3.77	0.098	0.25	0.59	0.37
Crude fat	0.803	0.784	0.733	0.735	0.0197	<0.001	0.44	0.32
ME, MJ/d	250	247	250	242	5.7	0.42	0.044	0.39
AA intake, g/d								
Arg	152	161	183	172	4.2	<0.001	0.62	<0.001
His	61.5	59.6	62.6	58.8	1.52	0.96	0.002	0.23
Ile	130	137	130	134	3.3	0.33	0.003	0.36
Leu	230	239	230	234	5.8	0.42	0.040	0.34
Lys	134	134	141	135	3.4	0.036	0.067	0.062
Met	47.1	48.6	38.5	43.4	1.14	<0.001	<0.001	0.010
Phe	149	153	149	149	3.7	0.31	0.24	0.39
Thr	126	128	116	121	3.1	<0.001	0.033	0.51
Trp	37.0	37.4	33.3	34.8	0.90	<0.001	0.051	0.24
Val	169	175	163	169	4.2	0.008	0.014	0.84
Σ Branched AA	529	551	523	536	13.3	0.14	0.015	0.48
Σ EAA	1,236	1,273	1,247	1,250	31.2	0.72	0.20	0.27
Σ NEAA[3]	1,503	1,529	1,481	1,484	37.5	0.082	0.45	0.54
Σ Total AA[4]	2,739	2,802	2,728	2,734	68.7	0.26	0.32	0.41
Total-tract apparent digestibility, g/kg								
DM	729	732	743	735	3.2	0.025	0.43	0.13
OM	747	751	760	752	3.4	0.045	0.57	0.13
NDF	696	705	696	705	11.6	0.99	0.44	0.99
Starch	960	963	965	963	0.8	0.007	0.30	0.006
CP	671	674	691	680	5.6	0.034	0.51	0.21
Yield								
Milk, kg/d	31.0	30.0	28.5	29.7	0.53	<0.001	0.86	0.002
ECM, kg/d	34.1	33.8	32.3	32.9	0.86	0.006	0.70	0.31
Fat, g/d	1,469	1,483	1,414	1,433	51.9	0.074	0.56	0.95
Protein, g/d	1,127	1,106	1,050	1,070	23.4	<0.001	0.96	0.058
Lactose, g/d	1,383	1,328	1,276	1,320	27.0	0.002	0.72	0.005
Milk composition								
Fat, %	4.74	4.94	4.92	4.81	0.146	0.70	0.57	0.042
Protein, %	3.65	3.71	3.69	3.62	0.080	0.20	0.92	0.004
Lactose, %	4.46	4.42	4.46	4.44	0.036	0.68	0.34	0.83
MUN, mg/L	126	135	141	138	7.1	<0.001	0.12	0.008
ME balance, MJ/d	3.76	1.58	13.40	1.58	6.123	0.054	0.009	0.054
ECM/DMI	1.47	1.48	1.39	1.47	0.044	0.018	0.007	0.044
ΔBCS[5]	−0.02	0.02	0.02	0.01	0.028	0.67	0.62	0.48

[1]RSM = rapeseed meal as a protein feed; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba beans as a protein feed; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed.

[2]BP = the effect of protein source in the basal diet (RSM + RSM-SPI vs. FB + FB-SPI); SPI = the effect of partial substitution of rapeseed meal and faba beans by spirulina (RSM + FB vs. RSM-SPI + FB-SPI); BP × SPI = interaction between the protein source in the basal diet and its substitution by spirulina.

[3]Includes Ala, Asp, Cys, Glu, Gly, Pro, Ser, and Tyr.

[4]Σ EAA + Σ NEAA.

[5]Change in BCS, units.

when RSM was substituted by spirulina, but remained unchanged with FB-supplemented diets ($P < 0.001$ for interaction).

Treatments had no effect on arterial concentrations of plasma energy metabolites (Table 5). Mammary plasma flow (L/d and L/kg milk) was increased in cows on RSM but decreased in cows on FB when spirulina was included ($P < 0.05$ for interaction; Table 6). Mammary uptake of acetic acid ($P < 0.05$) was increased when RSM was substituted by FB.

Amino Acid Metabolism

Faba beans resulted in lower arterial concentrations of taurine ($P < 0.001$), tyrosine ($P < 0.001$), ornithine ($P < 0.05$), Nπ-methylhistidine ($P < 0.05$), and all other EAA ($P < 0.05$) except threonine in comparison to RSM-supplemented diets (Table 5). A tendency for lower concentration of cysteine ($P = 0.092$) on FB than on RSM-supplemented diet was also observed. Arterial concentrations of tryptophan and Nτ-methylhistidine were decreased in cows on RSM but increased in those on FB when spirulina was included ($P < 0.05$ for interaction).

Mammary uptake of glutamine ($P < 0.01$) was increased when RSM was substituted by FB (Table 6). The opposite was true for methionine ($P < 0.05$) and phenylalanine ($P = 0.001$). A tendency for lower mammary uptake of histidine and leucine in cows on FB than those on RSM-supplemented diets was also observed ($P = 0.069$ and $P = 0.058$, respectively). Mammary uptake of methionine was decreased when

Table 4. Effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on rumen fermentation characteristics and N metabolism in lactating cows

	Treatment[1]					Orthogonal contrast[2]			
Item	RSM	RSM-SPI	FB	FB-SPI	SEM	BP	SPI	BP × SPI	D × T
pH	6.26	6.16	6.28	6.16	0.042	0.81	0.019	0.80	0.047
NH_4-N, mmol/L	6.12	8.54	9.37	8.43	0.681	0.003	0.102	0.002	0.12
VFA total, mmol/L	102	104	100	106	2.3	0.90	0.096	0.30	0.13
Molar proportions, mmol/mol									
Acetate	679	670	670	668	1.9	0.011	0.007	0.087	0.81
Propionate	166	174	169	175	3.4	0.40	0.027	0.70	0.83
Butyrate	123	123	126	122	2.7	0.61	0.44	0.57	0.62
Isobutyrate	8.91	9.35	10.1	9.63	0.222	<0.001	0.96	0.020	0.42
Valerate	12.6	13.0	13.0	13.1	0.27	0.22	0.28	0.55	0.16
Isovalerate	4.46	5.17	5.58	5.58	0.125	<0.001	0.007	0.008	0.60
Caproate	6.12	6.24	6.36	6.28	0.335	0.50	0.92	0.65	0.52
Molar ratios									
Acetate/propionate	4.13	3.88	4.00	3.84	0.084	0.21	0.013	0.49	0.67
(Acetate + butyrate)/propionate	4.87	4.59	4.75	4.54	0.110	0.31	0.018	0.64	0.66
N intake, g/d	617	620	615	604	15.7	0.26	0.57	0.39	
Ruminal microbial N flow, g/d	389	390	381	406	10.8	0.55	0.101	0.13	
Excretion in milk									
Milk N, g/d	177	173	165	168	3.7	<0.001	0.97	0.058	
Milk N:N intake, %	29.0	28.1	26.8	27.9	0.82	0.007	0.76	0.023	
Milk N:human edible N intake, %	143	103	83.7	84.1	3.34	<0.001	<0.001	<0.001	
Excretion in urine									
Urine, L/d	32.2	31.7	31.4	32.2	0.91	0.98	0.84	0.29	
Allantoin, mmol/d	483	482	475	504	10.4	0.49	0.15	0.13	
Uric acid, mmol/d	64.2	67.1	64.5	69.2	6.53	0.49	0.036	0.61	
Total purine derivatives, mmol/d	547	549	539	573	15.1	0.47	0.101	0.15	
Urinary urea N, g/d	113	124	129	127	5.0	0.020	0.24	0.100	
Urinary N, g/d	200	219	215	223	7.4	0.045	0.007	0.28	
Urinary urea N:urinary N, %	56.1	56.6	59.8	56.5	1.47	0.17	0.28	0.15	
Urinary N:N intake, %	32.3	35.3	35.1	37.1	0.90	0.008	0.005	0.58	
Excretion in feces									
Fecal N, g/d	203	202	189	193	6.7	0.027	0.77	0.59	
Fecal N:N intake, %	32.9	32.6	30.9	32.0	0.58	0.028	0.43	0.23	
Total fecal and urinary N excretion, g/d	402	420	404	417	12.7	0.88	0.045	0.71	
N balance, g/d	38.3	26.1	46.4	19.2	7.17	0.93	0.010	0.28	

[1]RSM = rapeseed meal as a protein feed; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba beans as a protein feed; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed.

[2]BP = the effect of protein source in the basal diet (RSM + RSM-SPI vs. FB + FB-SPI), SPI = the effect of partial substitution of rapeseed meal and faba beans by spirulina (RSM + FB vs. RSM-SPI + FB-SPI), BP × SPI = interaction between the protein source in the basal diet and its substitution by spirulina, and D × T = interaction between diet and sampling time.

spirulina substituted half of the protein in RSM and FB in the diet ($P < 0.01$).

DISCUSSION

Composition of Protein Feeds

The chemical composition of spirulina with high CP and methionine concentration, low histidine concentration, and no detectable NDF is in agreement with our previous experiments (Lamminen et al., 2017, 2019). However, the pore size of crucibles used in NDF analysis seems to affect greatly the results on microalgae NDF concentration (Lamminen et al., 2019). The starch concentration of FB was around 10-fold higher than that of RSM and almost 50% of the starch concentration in barley. In contrast, the NDF concentration of FB was only half that of RSM, and only 2% of the NDF in FB was indigestible, whereas this value was 53% in RSM. These values are in good agreement with Ramin et al. (2017). Similarly, the AA concentration with very low levels of methionine and moderate levels of histidine is typical for FB (Aguilera et al., 1992; Duc et al., 1999). The histidine concentration of RSM was slightly lower in the current experiment than in our previous experiments (Lamminen et al., 2017).

Feed Intake, Digestibility, and Milk Production

The DMI did not differ between RSM- and FB-supplemented diets, which agrees with Ingalls and McKirdy (1974) and Ramin et al. (2017). In Puhakka et al. (2016), the substitution of RSM with FB linearly decreased silage and total DMI, and the effect was greater at the high than the low CP level (15.4 and 19.0% in DM). In contrast to our hypothesis, DMI was decreased 0.65 kg/d and 2.8% by spirulina inclusion in the diet. Previously, when cows were able to choose between microalgae-containing concentrates and grass silage in separate feeding, the total DMI was not affected, but the concentrate intake was decreased as much as 2.1 kg/d when microalgae substituted conventional protein feeds (Lamminen et al., 2017, 2019). Therefore, it seems that TMR feeding cannot solve the feed intake problems related to feed use of microalgae, even though it has been reported to decrease sorting behavior of animals compared with separate feeding systems, thus leading to more balanced nutrient intake (DeVries and von Keyserlingk, 2009; Greter et al., 2010). The poor palatability of microalgae may be related to its sensory attributes as discussed by Lamminen et al. (2019). The avoidance of feeds rich in sulfuric (garlic, meaty, and fishy aromas) and terpenic (solvent, spice, and wood

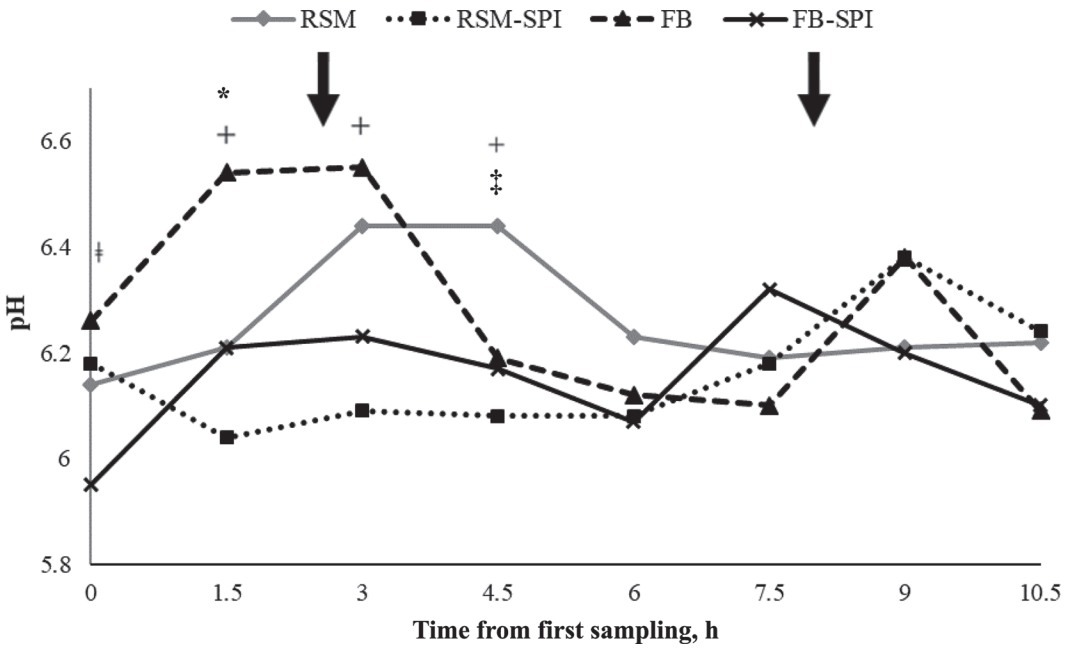

Figure 1. Effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on rumen pH. RSM = rapeseed meal as a protein feed; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba beans as a protein feed; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed. Average values are shown in relation to sampling time, and different symbols indicate a significant effect of the protein source in the basal diet (*), effect of partial substitution of rapeseed meal and faba beans by spirulina (+), and interaction between the protein source in the basal diet and its substitution by spirulina (‡) at $P < 0.05$ at a certain sampling time. Black arrows at the top of the figure indicate feeding times of TMR. SEM at time 0 (SEM0), 0.060; SEM1.5, 0.069; SEM3, 0.095; SEM4.5, 0.071; SEM6, 0.121; SEM7.5, 0.082; SEM9, 0.132; and SEM10.5, 0.124.

Table 5. Effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on arterial concentrations of plasma metabolites, AA and carnosine in lactating cows

	Treatment[1]					Orthogonal contrast[2]		
Item	RSM	RSM-SPI	FB	FB-SPI	SEM	BP	SPI	BP × SPI
Plasma metabolites								
Acetic acid, mmol/L	1.48	1.37	1.48	1.50	0.059	0.22	0.37	0.19
BHB, mmol/L	0.810	0.733	0.763	0.768	0.0421	0.85	0.23	0.18
Glucose, mmol/L	3.48	3.55	3.52	3.56	0.081	0.52	0.25	0.76
Insulin, μIU/mL	17.6	19.7	19.7	19.2	2.30	0.56	0.58	0.41
NEFA,[3] mmol/L	0.105	0.108	0.096	0.100	0.0107	0.28	0.66	0.97
EAA, μmol/L								
Arg	77.2	72.2	67.2	65.9	3.90	0.003	0.17	0.42
His	36.7	39.2	32.3	33.0	3.29	0.048	0.53	0.74
Ile	112	118	113	112	5.2	0.047	0.46	0.58
Leu	120	122	105	106	4.8	<0.001	0.57	0.99
Lys	95.9	91.9	86.8	86.3	4.92	0.014	0.40	0.52
Met	23.9	23.3	20.8	20.7	1.19	0.007	0.66	0.74
Phe	45.0	44.6	41.0	40.7	1.68	<0.001	0.67	0.97
Thr	104	101	97.9	105	4.65	0.70	0.47	0.101
Trp	33.3	30.9	30.1	31.0	1.29	0.064	0.32	0.049
Val	235	230	210	215	8.1	<0.001	0.95	0.35
NEAA, μmol/L								
Ala	231	229	227	233	12.1	0.98	0.79	0.55
β-Ala	4.02	3.78	3.87	3.80	0.198	0.58	0.22	0.52
Asn	45.1	43.4	44.7	44.1	1.570	0.91	0.40	0.66
Asp	2.94	4.15	3.91	5.07	0.543	0.105	0.046	0.97
Cit	59.2	58.1	59.9	61.7	2.28	0.15	0.79	0.32
Cys	21.3	21.0	19.5	20.6	0.81	0.092	0.57	0.26
Glu	83.3	78.3	85.3	86.7	2.99	0.036	0.45	0.18
Gln	245	245	252	241	10.0	0.79	0.36	0.34
Gly	287	291	308	337	18.3	0.003	0.090	0.22
Nτ-Methylhistidine[4]	3.42	3.23	3.14	3.36	0.262	0.34	0.87	0.015
Nπ-Methylhistidine[4]	3.85	3.68	3.38	3.52	0.152	0.014	0.91	0.19
Orn	44.6	41.7	39.9	40.7	2.65	0.030	0.38	0.14
Pro	74.5	70.3	70.7	70.9	3.74	0.38	0.29	0.25
Ser	96.0	95.2	104	108	5.86	0.017	0.70	0.54
Taurine	32.2	31.3	27.3	28.4	1.81	<0.001	0.97	0.46
Tyr	43.7	42.2	37.5	38.2	2.46	<0.001	0.79	0.41
Σ Branched AA	478	470	427	433	17.1	<0.001	0.90	0.53
Σ EAA	894	873	803	815	27.7	<0.001	0.80	0.36
Σ NEAA[5]	1,129	1,119	1,153	1,183	37.3	0.104	0.69	0.45
Σ Total AA[6]	2,023	1,992	1,956	1,999	55.6	0.44	0.89	0.36
Carnosine	21.3	21.9	20.5	20.3	1.32	0.23	0.85	0.71

[1]RSM = rapeseed meal as a protein feed; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba beans as a protein feed; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed.

[2]BP = the effect of protein source in the basal diet (RSM + RSM-SPI vs. FB + FB-SPI); SPI = the effect of partial substitution of rapeseed meal and faba beans by spirulina (RSM + FB vs. RSM-SPI + FB-SPI); BP × SPI = interaction between the protein source in the basal diet and its substitution by spirulina.

[3]NEFA = nonesterified fatty acids.

[4]IUPAC nomenclature: Nτ-Methylhistidine = the product of muscle actin and myosin catabolism; Nπ-Methylhistidine = the product of anserine breakdown.

[5]Includes Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr.

[6]Σ EAA + Σ NEAA.

aromas) compounds has been reported in sheep (Rapisarda et al., 2012). The aroma profile of microalgae varies depending on species, with some having fishy and seafood-like odor characteristics, but it can also be affected by oxidation of lipids and degradation of various other compounds (Van Durme et al., 2013). However, dietary mineral concentration and especially electrolytes Na and K have also been linked to decreases in DMI via their potential to increase osmolality of rumen fluid (Allen, 2000). For spirulina, Na concentrations of 1.2 to 18 g/kg DM have been reported (Ortega-Calvo et al., 1993; Costa et al., 2016), which largely exceed the Na concentrations of RSM and FB (0 to 0.5 g/kg DM; Heuzé et al., 2018a, b). The K concentration of spirulina (14–16 g/kg DM; Ortega-Calvo et al., 1993; Costa et al., 2016) is relatively similar to that of RSM and FB (9.5–15 g/kg DM; Heuzé et al., 2018a, b). To overcome palatability problems of microalgae diets,

Table 6. Effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on mammary uptake of plasma metabolites, AA, and carnosine in lactating cows

	Treatment[1]					Orthogonal contrast[2]		
Item	RSM	RSM-SPI	FB	FB-SPI	SEM	BP	SPI	BP × SPI
Mammary plasma flow								
L/d	15,755	18,377	18,860	17,532	1,123.3	0.22	0.48	0.041
L/kg of milk	507	608	657	590	33.7	0.038	0.57	0.011
Plasma metabolites								
Acetic acid, mol/d	13.7	12.8	17.6	16.6	1.74	0.017	0.51	0.97
BHB, mol/d	4.37	4.02	4.69	5.08	0.576	0.16	0.97	0.44
Glucose, mol/d	13.3	13.9	14.3	14.8	0.793	0.27	0.49	0.95
NEFA,[3] mol/d	−0.349	−0.368	−0.461	−0.321	0.1128	0.72	0.51	0.39
EAA, mmol/d								
Arg	516	515	469	521	30.0	0.51	0.42	0.39
His	221	239	181	194	23.7	0.069	0.50	0.91
Ile	717	695	723	678	39.5	0.89	0.42	0.78
Leu	974	988	922	899	34.5	0.058	0.90	0.61
Lys	816	847	837	829	37.8	0.97	0.77	0.62
Met	209	194	196	186	7.0	0.026	0.008	0.56
Phe	353	348	321	326	6.76	0.001	1.00	0.40
Thr	466	485	474	532	26.8	0.20	0.072	0.36
Trp	79.1	92.0	86.2	98.7	8.48	0.41	0.14	0.98
Val	926	943	859	877	40.1	0.12	0.67	1.00
NEAA, mmol/d								
Ala	379	307	420	438	89.7	0.16	0.65	0.44
β-Ala	10.5	9.62	12.5	8.82	1.922	0.76	0.26	0.48
Asn	250	237	242	246	11.2	0.98	0.65	0.36
Asp	12.2	34.2	30.8	43.3	7.66	0.091	0.039	0.56
Cit	58.5	59.8	65.3	94.3	27.17	0.46	0.59	0.62
Cys	27.4	25.8	21.5	26.3	3.81	0.49	0.69	0.41
Glu	489	395	573	569	53.8	0.006	0.24	0.27
Gln	1,433	1,426	1,484	1,364	98.1	0.95	0.47	0.52
Gly	99.2	227	223	395	88.0	0.12	0.11	0.81
Nτ-Methylhistidine[4]	−0.62	−2.73	−2.73	−1.11	0.950	0.80	0.80	0.065
Nπ-Methylhistidine[4]	5.19	5.10	0.24	1.50	2.002	0.050	0.78	0.75
Orn	310	324	324	338	18.0	0.44	0.43	1.00
Pro	184	166	188	190	17.0	0.31	0.54	0.46
Ser	529	493	425	514	48.5	0.41	0.59	0.22
Taurine	47.0	70.4	39.0	48.4	9.97	0.16	0.13	0.50
Tyr	331	324	317	324	12.1	0.40	0.98	0.37
Σ Branched AA	2,617	2,626	2,503	2,453	107.6	0.21	0.86	0.79
Σ EAA[5]	5,277	5,347	5,023	5,140	202.3	0.25	0.64	0.91
Σ NEAA[6]	3,734	3,634	3,923	4,110	245.5	0.11	0.83	0.47
Σ Total AA	9,011	8,981	8,970	9,250	306.5	0.67	0.64	0.56
Carnosine	33.5	52.0	16.7	9.46	14.99	0.067	0.72	0.41

[1]RSM = rapeseed meal as a protein feed; RSM-SPI = mixture of rapeseed meal and *Spirulina platensis* (1:1 on CP basis) as a protein feed; FB = faba beans as a protein feed; FB-SPI = mixture of faba beans and *Spirulina platensis* (1:1 on CP basis) as a protein feed.

[2]BP = the effect of protein source in the basal diet (RSM + RSM-SPI vs. FB + FB-SPI); SPI = the effect of partial substitution of rapeseed meal and faba beans by spirulina (RSM + FB vs. RSM-SPI + FB-SPI); BP × SPI = interaction between the protein source in the basal diet and its substitution by spirulina.

[3]NEFA = nonesterified fatty acids.

[4]IUPAC nomenclature: Nτ-Methylhistidine = the product of muscle actin and myosin catabolism; Nπ-Methylhistidine = the product of anserine breakdown.

[5]Includes Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

[6]Σ EAA + Σ NEAA.

further research is warranted on the feeding strategies and processing of microalgae.

The digestibility of DM, OM, and CP were increased when RSM was substituted by FB, which is consistent with the higher digestibility values of OM (Heuzé et al., 2018a,b; Luke, 2019), CP, and NFE (Luke, 2019) in FB than RSM. This is likely attributable to the lower lignin concentration in FB than in RSM (Heuzé et al., 2018a,b), which is supported by our results on indigestible NDF concentration. Puhakka et al. (2016) found that the digestibility responses to substituting RSM with FB depended on the dietary CP level. In their experiment, the apparent digestibility of DM and OM increased linearly and CP quadratically with a high CP level but remained unchanged with a low CP level when RSM was substituted with FB. In the current

experiment, the apparent total-tract digestibility of DM, OM, NDF, and CP was not affected by spirulina inclusion in the diet, similar to our previous experiments comparing microalgae and RSM (Lamminen et al., 2017) and soybean meal (Lamminen et al., 2019). It is important to note that the results may have been affected to some extent by the proportion of barley in the rations, which was used to adjust the concentrate-to-forage ratio in TMR.

In agreement with our hypothesis, the complete substitution of RSM by FB decreased ECM and protein yields, suggesting poorer value of FB protein than rapeseed protein for milk production. The same was observed in Puhakka et al. (2016). In Ramin et al. (2017), the substitution of rapeseed expeller by FB or pea resulted in decreased milk, protein, and lactose yields. Also as hypothesized, milk production response to spirulina inclusion in the diet differed between the 2 protein sources in the basal diets. With RSM-supplemented diets, the decrease in milk production by spirulina inclusion in the diet was likely mainly caused by the decreases in DMI rather than unbalanced AA profile because feed conversion efficiency (ECM:DMI ratio) was maintained at a similar level when spirulina substituted half of the protein in RSM. This finding contradicts the results of our previous experiment in which the lowest feed conversion efficiency was found with a mixture of spirulina and RSM in comparison to diets having exclusively either spirulina or RSM as protein feeds (Lamminen et al., 2017). On the other hand, no difference in feed conversion efficiency was found in the experiment in which a mixture of spirulina and *Chlorella vulgaris* substituted the protein of RSM in half or totally (Lamminen et al., 2017). Nevertheless, in the current experiment, the milk production response to partial substitution of RSM by spirulina was in agreement with the common perception of DMI being the main determinant of milk production (Hristov et al., 2004). This outcome also suggests that the milk production response of spirulina diets may be boosted if palatability problems related to microalgae feeding could be solved (e.g., by feed processing).

Despite of the decrease in DMI in cows on FB-supplemented diets due to spirulina, milk production was increased. This increase was likely caused by improved quality of protein supply, such as more balanced AA supply or lower ruminal protein degradability, as indicated by increased feed conversion efficiency by spirulina inclusion in the FB-supplemented diets.

Notably, in spite of the different milk yield response to spirulina with RSM- and FB-supplemented diets, spirulina inclusion in the diet did not affect ECM yields differently. The changes in milk yield were counteracted by changes in milk component concentrations induced by spirulina inclusion in the diet, resulting in ECM yields being unaffected. Milk fat and protein concentrations were increased with decreasing milk yield when spirulina substituted RSM, and vice versa for FB-supplemented diets. This finding is in agreement with the notion that milk protein and fat concentration are typically negatively correlated with milk yield (Oldham and Sutton, 1980). However, the increase in milk fat concentration by spirulina inclusion in the RSM-supplemented diet was proportionally much larger than that of protein concentration. The result may be related to the characteristics of spirulina, as in our earlier experiment (Lamminen et al., 2019) the substitution of soybean meal by microalgae increased milk fat concentration especially with spirulina. Milk fat concentration has often increased with postruminal infusion of methionine (Varvikko et al., 1999; Zanton et al., 2014) but also with dietary supplementation of methionine sources (Zanton et al., 2014). Varvikko et al. (1999) also reported increasing ECM yields with increasing postruminal infusion of methionine. However, Haque et al. (2012) reported that the balancing of duodenal supply of AA increased milk and protein yield and milk protein concentration, but decreased milk fat concentration irrespective of the dietary CP concentration.

Energy and Nitrogen Metabolism

All experimental diets were sufficient in N supply for microbial needs as judged by MUN concentrations exceeding the concentration of 117 mg/L that has been suggested for optimally balanced degradable N:ME ratio of the diets (Nousiainen et al., 2004). Despite similar N intake, the concentration of MUN and ruminal NH_4-N were increased when RSM was substituted with spirulina or FB, and decreased when FB were substituted with spirulina. This outcome suggests that the order of ruminal protein degradability of these protein feeds was in the following sequence of decreasing degradability: FB > spirulina > RSM. This ordering is in agreement with our previous experiments comparing RSM and spirulina (Lamminen et al., 2017). It is also supported by the 17- to 20-percentage-unit higher ruminal protein degradability of FB than that of RSM reported in feed tables (Heuzé et al., 2018a,b; Luke, 2019). In addition, MUN concentrations were increased when rapeseed was isonitrogenously substituted by FB (Puhakka et al., 2016), and when ruminal degradability of rapeseed protein was increased in isonitrogenous diets (Mutsvangwa et al., 2016). This was also accompanied with increasing ruminal NH_4-N concentrations, but no effect was observed on NUE (milk N:N intake) (Mutsvangwa et al., 2016). In the current experiment,

NUE was decreased, and the proportion of N excreted in urine increased when RSM was substituted by FB. However, total N excretion was not affected. The degradability of CP alone seems to be poor predictor of NUE (Huhtanen and Hristov, 2009), thus the observed decrease in NUE may have been caused by the unbalanced AA profile of FB rather than the degradability of protein. When spirulina substituted half of the protein in RSM, NUE was decreased, whereas the opposite was true for FB-supplemented diets. This probably reflects poorer balance of AA in spirulina than in RSM but complementary AA composition of spirulina and FB. It might also be possible that this finding was to some extent influenced by the efficiency of ruminal microbial synthesis because microbial N flow was numerically higher with FB-SPI than FB but remained unaffected with rapeseed diets (interaction $P = 0.13$).

Human-edible protein efficiency of milk production measures the efficiency of conversion of N in human-edible products into milk N. When human-edible efficiency is >100%, the animal production contributes to food supply; that is, it generates more human-edible products than is consumed in feed (Baldwin, 1984). Rapeseed meal–supplemented diets resulted in the highest human-edible protein efficiency compared with other diets, and they were the only diets in the current experiment increasing the return on human-edible inputs. The situation may change, however, if food use of rapeseed protein increases in the future or processing of microalgae and FB generates high-quality protein by-products inedible to human consumption. In general, the human-edible protein efficiency was rather low in the current experiment, and the results contradict the review of Dijkstra et al. (2013). That review summarized the results of several studies on human-edible protein efficiency of milk production, which were in all cases well over 100% (141 to 1,430%); that is, milk production was always the net contributor of the human-edible food supply. These very high efficiencies are only achievable on diets high in forage. However, the results on FB-supplemented diets with human-edible efficiency of 84% is in agreement with Karlsson et al. (2018) reporting an efficiency of 73% for a grass silage–based diet supplemented with cereal grain and soybean meal. They reported efficiencies up to 268% when dietary concentrates were constituted completely of by-products (RSM, sugar beet pulp, and dried distillers grains with solubles).

Ruminal fermentation pattern in the current experiment was characterized by a high molar proportion of acetate and a low molar proportion of propionate, which is typical for diets based on restrictively fermented grass silage (Huhtanen, 1998). The slightly decreased molar proportion of acetate in the rumen VFA when RSM was substituted by FB may reflect lower NDF and higher starch concentration in FB-supplemented than RSM-supplemented diets. The responses of molar proportions of isobutyrate and isovalerate to spirulina inclusion in the diet depended on the source of protein in the basal diet. These branched chain VFAs originate from microbial fermentation of valine and leucine (Allison, 1978). Indeed, with RSM the response to spirulina inclusion with increasing proportion of isobutyrate and isovalerate was in line with the increasing intakes of valine and leucine. However, with the FB-supplemented diet, the proportions of these branched chain VFAs were decreased or remained unaffected despite the increased intake of valine and leucine when FB were partially substituted by spirulina. This response may be caused by the lower proportion of rumen degradable protein in spirulina than in FB, leading to decreased supply of valine and leucine to rumen microbes when FB were partially substituted by spirulina. Mutsvangwa et al. (2016) also observed decreasing ruminal concentrations of isobutyrate and isovalerate when ruminal degradability of isonitrogenous RSM-supplemented diets was decreased with micronization.

Total ruminal VFA production tended to increase with spirulina inclusion in the diet, which contradicts the in vitro results of Wild et al. (2019) reporting low ruminal fermentation and microbial N production with several microalgae species. Moreover, the ratio of acetate and butyrate to propionate was decreased when FB and RSM were substituted by spirulina. The lowered proportions of lipogenic VFA suggest that the observed opposite changes in milk fat concentration by spirulina inclusion in the diet were not caused by ruminal fermentation, but may be related to other factors such as a dilution effect or protein characteristics of spirulina, as already discussed. Despite palatability problems with spirulina diets, dietary treatments likely had no major effect on the energy metabolism of dairy cows based on positive ME balances and unaffected arterial concentrations of plasma metabolites, and BCS.

Amino Acid Metabolism

Results of AA metabolism were affected by both AA profile and ruminal protein degradability of the experimental protein feeds. Histidine and methionine are important AA to consider for diets supplemented by FB and spirulina because spirulina is low in histidine but rich in methionine (Lamminen et al., 2017, 2019), whereas the opposite is true for FB, similar to many grain legumes (Aguilera et al., 1992; Halmemies-Beauchet-Filleau et al., 2018). Methionine may limit milk production in cows on FB-supplemented diets (Puhakka et al., 2016), and histidine is typically the

first limiting AA with diets based on cereal and grass silage (Kim et al., 1999; Vanhatalo et al., 1999). Being high in both histidine and methionine (Maxin et al., 2013), rapeseed is therefore well suited to supplement diets based on cereal and grass silage.

Irrespective of the intake of EAA, the arterial concentrations of most EAA were decreased when RSM was substituted by FB. This decrease was presumably caused by lower intestinal absorption of AA, which was evident by decreased plasma concentration of BCAA when RSM was substituted with FB despite unaffected intake. Changes in plasma BCAA concentration can be used as an indicator of AA absorption from the small intestine (Bergen et al., 1973) because in contrast to other AA, they are not extensively degraded in the liver after absorption (Harper et al., 1984). The lower intestinal availability of AA from FB than RSM was likely caused by higher ruminal protein degradability, which likely further reduced the already low methionine supply from FB-supplemented diets. The changes observed in arterial methionine, cysteine, serine, and taurine concentrations between RSM- and FB-supplemented diets are classical metabolic responses for altered dietary methionine supply. In the mammalian body, cysteine is produced irreversibly via the transsulfuration pathway from methionine, serine is used up in this conversion, and the cysteine produced is further metabolized to taurine (Finkelstein, 1990). Thus, an increased methionine supply typically increases the plasma concentrations of methionine, cysteine, and taurine and decreases that of serine (e.g., Titgemeyer and Merchen, 1990; Pisulewski et al., 1996), as was also observed in the current study between RSM- and FB-supplemented diets.

Spirulina inclusion in the diet resulted in 1.5 g/d and 4.9 g/d increases in methionine intake in cows on RSM- and FB-supplemented diets, respectively. The increased methionine supply may also explain the increased milk production response caused by spirulina inclusion in FB-supplemented diets. However, spirulina inclusion in the diet did not induce any changes in arterial methionine, cysteine, serine, or taurine concentrations. The same was true for our previous experiment comparing soybean meal and different microalgae species with an average 7.6 g/d higher methionine intake from microalgae-supplemented diets than soybean meal–supplemented diets (Lamminen et al., 2019). This outcome suggests that even though the intake of methionine was increased by microalgae inclusion in the diet, dietary AA supply did not convert to absorbed AA as in RSM-supplemented diets. This is further supported by the results of BCAA, the intake of which was increased by spirulina inclusion in the diet, but the plasma concentration of which remained unaffected. Because spirulina did not increase plasma BCAA concentration even in cows on the FB-supplemented diet despite the suggested lower protein degradability in spirulina than in FB, factors other than protein degradability likely contributed to the low intestinal absorption of AA from spirulina. Low intestinal digestibility of microalgal RUP is one explanation, as suggested by Wild et al. (2019).

Despite decreased histidine intake from spirulina inclusion in the diet, histidine likely had only a minor effect on animal performance in this experiment because the arterial concentrations of histidine and carnosine (an endogenic histidine reserve) and mammary uptake of histidine were unaffected by spirulina inclusion in the diet. This result is inconsistent with the results of our previous experiments (Lamminen et al., 2017) comparing rapeseed and spirulina or a mixture of spirulina and *Chlorella vulgaris*, in which the histidine supply may have been suboptimal with microalgae diets based on changes in arterial carnosine, mammary uptake of histidine, and milk protein yield. However, in these experiments, the histidine concentration of rapeseed supplement was 4.5 to 6.7 g/kg CP higher than in the RSM used in the current experiment, which likely further emphasized the differences between rapeseed and microalgae in Lamminen et al. (2017).

CONCLUSIONS

The current experiment verified the suitability of non-defatted spirulina as protein feed for lactating dairy cows. However, results suggest poorer palatability of spirulina than RSM and FB because DMI was decreased by spirulina inclusion in the diet, despite TMR feeding. As hypothesized, ECM yield was decreased when RSM was substituted completely by FB, which together with lower protein yield suggests poorer value of FB protein for milk production. This outcome is likely attributable to lower methionine concentration and higher ruminal degradability of FB than rapeseed protein. In agreement with our hypothesis, milk production response to spirulina inclusion in the diet depended on the source of protein in the basal diet. Based on feed conversion efficiency in cows on RSM-supplemented diets, the decrease of milk yield with spirulina inclusion in the diet was likely caused by lower DMI rather than protein or AA characteristics of spirulina. Contrasting milk production response in cows on FB most likely resulted from improved quality of protein feeding, such as increased methionine supply, improved AA balance in general, or lower ruminal protein degradability. The latter was evident from decreased MUN and ruminal NH_4-N concentrations when FB were partially substituted by spirulina. The unaffected ECM yield by spirulina inclusion in the diet is notable and was mainly attributable to the changes in milk fat concentration

and protein characteristics of spirulina. This study confirmed the lower protein yield response with FB than with rapeseed, and the results suggest complementary AA profiles of FB and spirulina. This suggestion implies a potential to improve milk production response from FB-supplemented diets with supplementation of methionine-rich feeds such as spirulina.

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